Journal of Neurochemistry, 2006, **97**, 201–210 doi:10.1111/j.1471-4159.2006.03723.x

The JAK3 inhibitor WHI-P154 prevents PDGF-evoked process outgrowth in human neural precursor cells

G. R. Richards,* A. J. Smith,* P. Cuddon,*,† Q. P. Ma,* M. Leveridge,* J. Kerby,* H. L. Roderick,† M. D. Bootman† and P. B. Simpson*

**The Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Harlow, Essex, UK*

†*Laboratory of Molecular Signalling, The Babraham Institute, Cambridge, UK*

Abstract

The prospect of manipulating endogenous neural stem cells to replace damaged tissue and correct functional deficits offers a novel mechanism for treating a variety of CNS disorders. The aim of this study was to investigate pathways controlling neurite outgrowth in human neural precursor cells, in particular in response to platelet-derived growth factor (PDGF). PDGF-AA, -AB and -BB were found to initiate calcium signalling and produce robust increases in neurite outgrowth. PDGF-induced outgrowth of Tuj1-positive precursors was abolished by the addition of EGTA, suggesting that calcium entry is a critical part of the signalling pathway. Wortmannin and PD098059 failed to inhibit PDGF-induced outgrowth. *Clostridium* Toxin B increased the amount of PDGF-induced neurite branching but had no effect on basal levels. In contrast, WHI-P154, an inhibitor of Janus protein tyrosine kinase (JAK3), Hck and Syk, prevented PDGF-induced neurite outgrowth. PDGF activates multiple signalling pathways with considerable potential for cross-talk. This study has highlighted the complexity of the pathways leading to neurite outgrowth in human neural precursors, and provided initial evidence to suggest that calcium entry is critical in producing the morphological changes observed.

Keywords: ArrayScan, human neural precursors, neurite outgrowth, platelet-derived growth factor, stem cells.

J. Neurochem. (2006) **97**, 201–210.

The application of human neural stem cells to CNS drug research offers an opportunity to develop *in vitro* cultures containing a mixture of neuronal, glial and astrocytic cell types which may provide a better reflection of the human brain than traditional recombinant cell lines (Simpson 2005). Such stem cell-derived cultures can be used to investigate signalling pathways relevant to a variety of disease states. In addition, identification of neural stem cells in the adult brain has led to increasing interest in the potential of using stem cells to treat CNS disorders, either by transplantation or manipulation of endogenous stem cells (for reviews see Mitchell *et al*. 2004; Zhu *et al*. 2005).

Whilst a large amount of work has focused on stem cell transplantation, less is known about the potential of stimulating endogenous stem cells to compensate losses of neural cells by injury or disease. Stem cells are known to divide, migrate and differentiate following injury to the adult brain (Magavi and Macklis 2002; Felling and Levison 2003), but they are unable to fully replace damaged tissue and correct functional deficits in normal circumstances. However, the potential to augment this endogenous response offers a novel mechanism for treating a variety of CNS disorders without many of the practical and ethical issues surrounding transplantation-based therapeutic strategies.

Five isoforms of platelet-derived growth factor (PDGF) have been identified to date: AA, AB and BB are formed by dimerization of A and B chains whilst the newer members, CC and DD, are secreted as inactive forms and less is known about their ability to dimerize (Tallquist and Kazlauskas

Received August 25, 2005; revised manuscript received November 1, 2005; accepted November 22, 2005.

Address correspondence and reprint requests to Gillian Richards, The Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, UK.
E-mail: gillian_richards@merck.com

Abbreviations used: CNTF, ciliary neurotrophic factor; CREB, cyclic-AMP responsive binding element protein; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; JAK, Janus protein tyrosine kinase; MAPK, mitogen activated protein kinase; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3′-kinase; PLC, phospholipase C; VIPRII, voltage-ion probe reader.

2004). Two PDGF receptors have been identified, α and β, which belong to the tyrosine kinase receptor family. PDGF binds to two receptors simultaneously, thus causing dimerization and tyrosine kinase activation. The α-receptor can bind both A and B chains, whilst the β-receptor binds only B chains (Heldin and Westermark 1999). Therefore, PDGF-AA can only activate α homodimers, PDGF-AB activates α homodimers or αβ heterodimers, and PDGF-BB has the broadest activity, producing αα, αβ or ββ dimers.

We have established *in vitro* human neural precursor cultures, suitable for drug discovery, from a commercial source of human neural precursors (Richards *et al*. 2004). This study describes a number of assays which have been developed to study cell morphology and signalling, and characterizes the signalling pathway underlying the response to a key neural growth factor, PDGF, in detail. In combination with other quantitative approaches for screening stem cell biology events (Richards *et al*. 2004), the studies performed here provide a model for the use of neural stem/precursor cells, and their progeny, within neuroscience drug discovery (Simpson 2005).

Methods

Cell culture

Human neural precursors were obtained from a commercial source (Batch no. 8F0389; Clonetics, Cambrex, East Rutherford, NJ, USA). According to the manufacturer's information, the neural precursor cells were obtained from cortical tissue from a female donor of 17 weeks gestation. Cells were supplied as neurospheres, characteristic of the cells produced by the methodology of Svendsen *et al*. (1998). Cells were grown as neurospheres in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Poole, UK)/F12 (Invitrogen Life Technologies, Paisley, UK) 3 : 1, 1% N2 (Invitrogen Life Technologies), epidermal growth factor (EGF) 20 ng/mL (Peprotech, London, UK) and leukemia inhibitory factor (LIF) 10 ng/mL (Chemicon, Chandlers Ford, Hampshire, UK), largely as previously described (Richards *et al*. 2004). Neurospheres were passaged every 2 weeks by chopping into quarters using a tissue chopper (Mickle Laboratory Engineering, Guildford, UK). Cells were thawed at passage 3 and were discarded after passage 20. Cells were prepared for plating 7–12 days post-chopping by Accutase (Innovative Cell Technologies, San Diego, CA, USA) dissociation. Briefly, this entailed washing neurospheres three times in phosphate-buffered saline (PBS, Sigma-Aldrich), adding 3 mL Accutase, diluted 1 : 5 with Hanks balanced salt solution (Invitrogen Life Technologies) and shaking for 30 min in a shaker-incubator at 37°C; 7 mL PBS were added, followed by trituration in a 10 mL pipette. Cells were harvested by centrifugation at 150 *g* for 5 min and resuspended in standard plating medium [DMEM/F12 3 : 1, transferrin 50 μg/mL (Merck Biosciences, Nottingham, UK)/insulin 5 μg/mL/progesterone 20 nM/putrescine 100 μM/T3 30 nM/selenium 30 nM (all from Sigma-Aldrich)] with further trituration. For microplate-based experiments, cells were plated at 17 500 cells per well in 96-well, black-sided, microtitre poly D-lysine-coated plates (Biocoat, BD Biosciences, Oxford, UK) coated with laminin 1 μg/mL (Sigma). For single cell calcium imaging studies, cells were plated, at 500 000 cells per well, in 6-well plates with a 22 mm glass coverslip coated with poly D-lysine and laminin in each well.

Culture characterization

Cells were plated in 96-well plates as described above. Cultures were maintained for varying periods of time, then fixed in ice-cold methanol for 3 min, washed three times in PBS, and blocked and permeabilized for 1 h with 5% normal goat serum/1% bovine serum albumin (BSA)/0.1% Triton-X 100 in PBS (all from Sigma-Aldrich). Immunofluorescence staining was performed using primary antibodies for 1 h at 25°C, and secondary antibodies and nuclear counterstain for 1 h at 25°C. Primary antibodies included 1 : 500 monoclonal anti-β3 tubulin ('Tuj1'; Covance, Cambridge Bioscience, Cambridge, UK), 1 : 200 polyclonal anti-nestin (Chemicon) and 1 : 1000 monoclonal anti-glial fibrillary acidic protein (GFAP) (Sigma-Aldrich). Alexa 488-conjugated goat anti-mouse secondary antibodies (1 : 500; Invitrogen Life Technologies) were used in conjunction with 30 μM Hoechst 33342. Cell counting was performed in an automated manner using a Cellomics ArrayScan II (Cellomics, Pittsburgh, PA, USA).

Neurite outgrowth

Growth factors were added to cells 1 h post-plating and incubated for 48 h at 37°C/5% CO_2. For mechanistic studies, inhibitors were added to cells 1 h post-plating, and PDGF-AB added following 10 min of incubation. Cell plates were then incubated for 48 h at 37°C/5% CO_2. Growth factors studied were PDGF-AA, -AB and -BB (all from Peprotech EC), ciliary neurotrophic factor (CNTF), acidic fibroblast growth factor (FGF) and basic FGF (all from Merck Biosciences). The inhibitors used were 30 μM WHI-P154, 50 nM wortmannin, 200 pg/mL *Clostridium difficile* Toxin B (all from Merck Biosciences) and 50 μM PD098059 (Sigma-Aldrich). Following incubation, cells were fixed in ice-cold methanol for 3 min, washed three times in PBS, and blocked and permeabilized for 1 h with 5% normal goat serum/1% BSA/0.1% Triton-X 100 in PBS (all from Sigma-Aldrich). Immunofluorescence staining was performed as described above.

A Cellomics ArrayScan II was used to automatically quantify process outgrowth as previously described (Simpson *et al*. 2001; Arden *et al*. 2002; Ramos Villullas *et al*. 2003). The ArrayScan automatically captures images of labelled nuclei and cellular processes, and applies an algorithm to measure process number, length and complexity. This protocol detects outgrowth from the human neural cell culture (Fig. 2a1–3). In this study, data are shown from two primary algorithm outputs (outgrowth index and form factor) and a daughter parameter derived from other algorithm outputs (average process length per cell). Outgrowth index indicates the percentage of cell with process lengths greater than the threshold level, and form factor provides an indication of the extent of process branching. In combination, these three parameters provide a robust indication of cell morphology.

Well-based microplate calcium imaging

Cells were washed with physiological salt solution (KHB: NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 4.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.2 mM, HEPES 10 mM; all from Sigma-Aldrich)

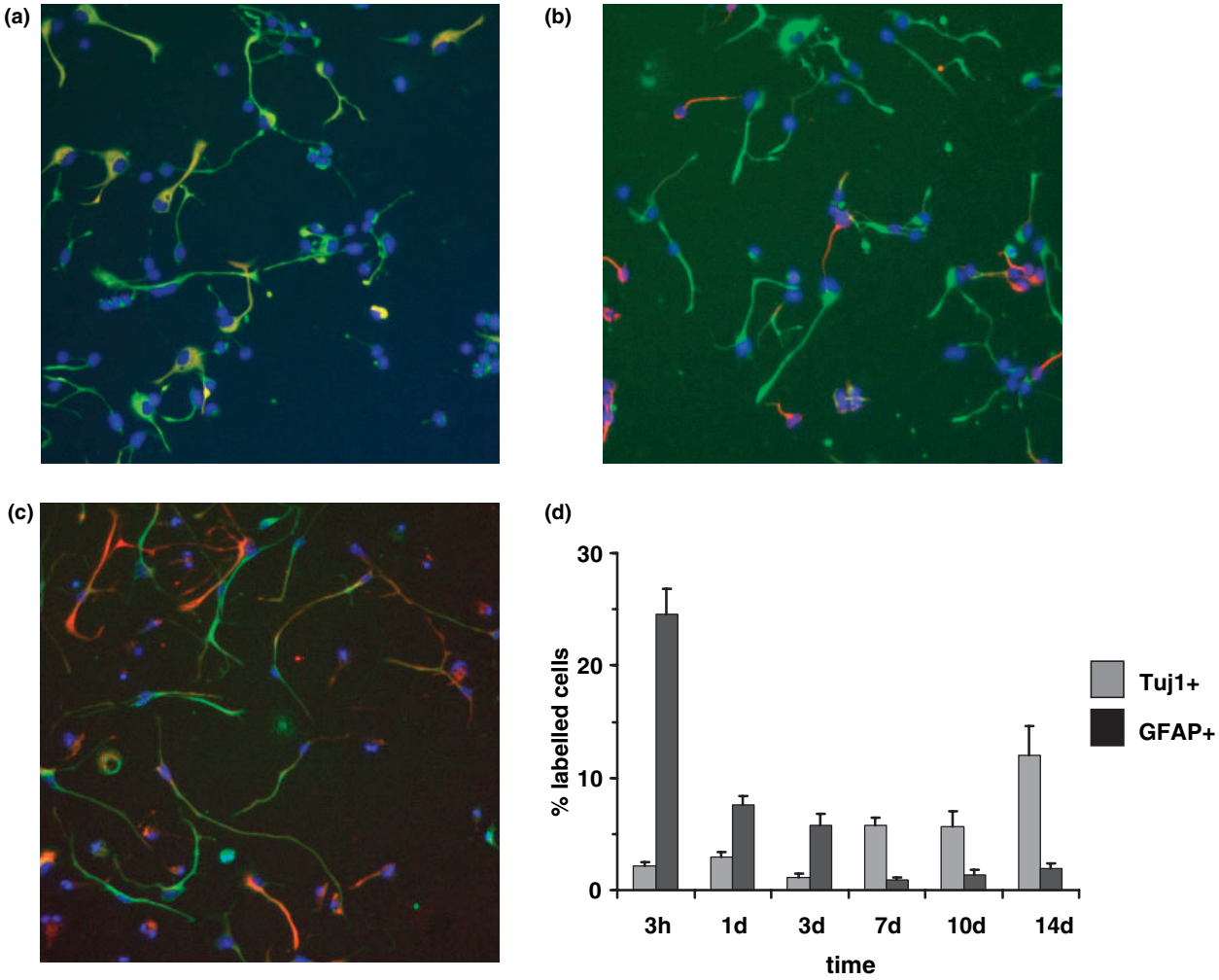

Fig. 1 Human neural precursors were plated, cultured and processed for immunofluorescence labelling experiments. Cells were dual labelled for (a) GFAP (red) and nestin (green) with dual labelling appearing in yellow, (b) Tuj1 (red) and nestin (green), and (c) GFAP (green) and Tuj1 (red). Nuclei are indicated in blue. (d) The proportion of GFAP+ cells decreased over a 15 day culture period, whereas the Tuj1+ cells increased to approximately 15% of the total cell population. $n = 7$ wells; error bars indicate standard error.

and incubated with 5 μM fluo-3AM/0.004% pluronic acid (Molecular Probes) for 1 h at 25°C. Following incubation, cells were washed three times in KHB and transferred to a voltage-ion probe reader (VIPRII; Aurora Discovery, San Diego, CA, USA) for compound addition and recording. Data were analysed using an in-house custom data analysis solution. Background fluorescence values were subtracted from the raw fluorescence values, which were then normalized to basal fluorescence. Data are hence expressed as 'change in fluorescence over basal' or ΔF/F.

Single-cell calcium imaging

Coverslips of cells were transferred to 35 mm dishes, washed in KHB three times and incubated with 5 μM fura-2AM (Molecular Probes) for 45 min in the dark at 25°C. Following incubation, coverslips were washed and transferred to an imaging system comprising an inverted microscope (Nikon, Kingston upon Thames, Surrey, UK), rapid solution changer (BioLogic, Claix, France), Deltaram excitation source, ICCD camera and IMAGEMASTER software (all supplied by Photon Technology International, Birmingham, NJ, USA). For some experiments, after recording PDGF responses, cells were processed for immunofluorescence *in situ*, using identical fixing and staining protocols as described above. Data were analysed in IMAGEMASTER using a macro to ratio the 340 nm : 520 nm and 380 nm : 520 nm images, followed by an in-house custom-designed data analysis solution to express data as 'change in fluorescence over basal' or ΔF/F.

Cell-based microplate calcium imaging

Cells were washed with KHB three times and incubated with 5 μM fluo-3AM/0.004% pluronic acid (Molecular Probes) for 1 h at 25°C. Following incubation, cells were washed three times in KHB, and transferred to a BD Pathway HT (BD Biosciences) plate imager for compound addition and image collection. After recording PDGF responses, cell plates were processed for immunofluorescence using fixing and staining protocols identical to those described above. Cell plates were returned to the BD Pathway HT for identification of the subpopulations of cells imaged in the kinetic experiments. Data

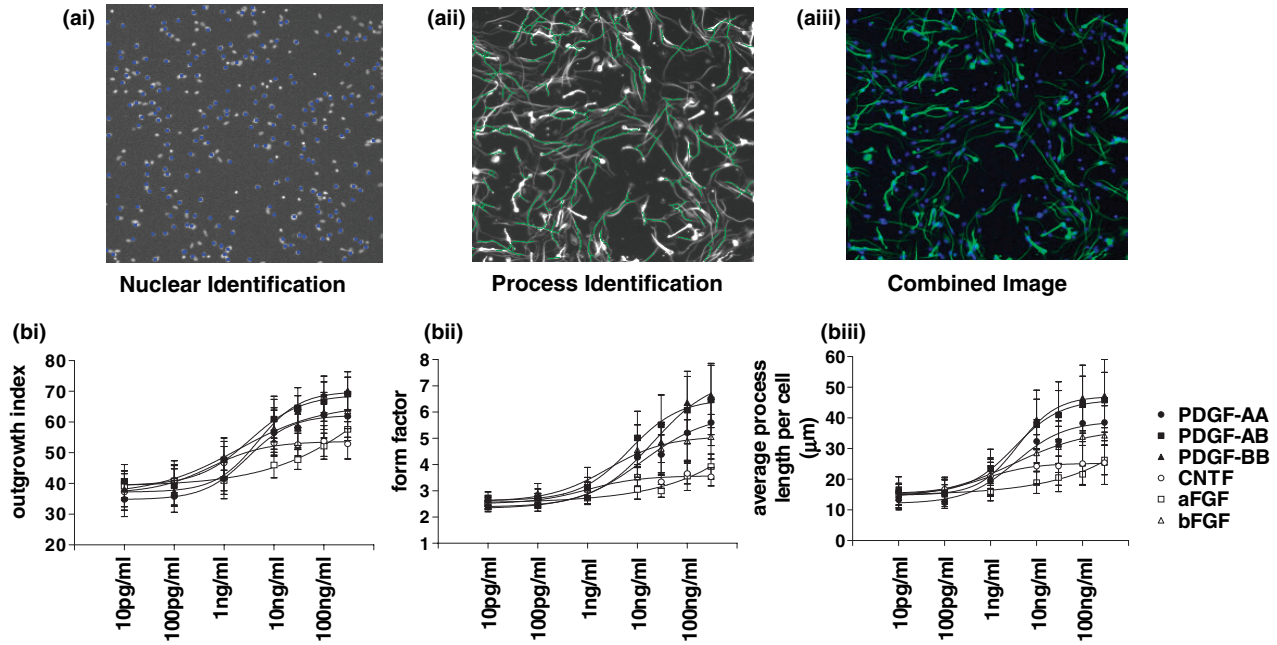

Fig. 2 (a) Cellomics ArrayScan automatically captures images of labelled nuclei (i) and cellular processes (ii), and a user-defined algorithm measures process number, length and complexity. The blue overlay indicates objects identified as nuclei on the basis of Hoechst 33342 labelling, and the green overlay indicates processes identified using anti-βIII tubulin primary antibody (Tuj1) and Alexa-488 conjugated secondary antibody. (b) PDGF-AA, -AB and -BB induce outgrowth in Tuj1-labelled cells, with increases in complexity and length of processes. Moderate outgrowth effects were elicited by bFGF, whilst aFGF and CNTF were without substantial effects. $n = 5$ separate experiments. The magnitude of effect was similar for all three PDGF isoforms.

were analysed using an in-house custom data analysis solution to express data as 'change in fluorescence over basal' or ΔF/F, and to quantify the amplitude, delay and area under the curve for PDGF calcium responses.

Statistics

Statistical analysis was performed using unpaired *t*-tests or one-way analysis of variance, as appropriate, within GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Cell culture characterization

Initial immunofluorescence labelling experiments were performed on cultured human neural cells derived from neurospheres. At 48 h after plating, a large proportion of the cells were positive for nestin, a marker for neural progenitor cells (Figs 1a and b, green-labelled cells). An overlapping subpopulation of these cells also labelled positive for GFAP, a marker for astrocytes (Fig. 1a, yellow labelling). A non-overlapping subpopulation of cells was found to express Tuj1, an early neuronal marker (Fig. 1b). No overlap in GFAP and Tuj1 labelling was observed (Fig. 1c). The proportion of GFAP- and Tuj1-positive cells in the cultures was followed over a 15 day culture period. Over this time course, initially approximately 25% of cells were GFAP+, decreasing to less than 5% at 15 days. The proportion of Tuj1+ cells increased from less than 5% to between 10 and 15% at 15 days (Fig. 1d). The cell cultures were examined for expression of a number of oligodendrocyte markers including O4, galactocerebroside C and myelin basic protein, none of which were found to be expressed (data not shown).

Differential effects of growth factors on neural cell process outgrowth

Following this immunocytochemical characterization of the cultures, we examined which growth factors would alter the morphology of the human neural cells. PDGF-AA, -AB and -BB induced a robust increase in outgrowth index in Tuj1-labelled neural cells in a concentration-dependent manner, following a 48 h incubation (PDGF-AA $EC_{50} = 4.7 \pm 1.2$ ng/mL; PDGF-AB $EC_{50} = 9.7 \pm 7.0$ ng/mL; PDGF-BB $EC_{50} = 6.0 \pm 0.9$ ng/mL; maximal effects observed at 300 ng/mL: see Fig. 2b1–3). Corresponding increases in process complexity and length were also observed. Moderate outgrowth effects were elicited by bFGF, whilst aFGF and CNTF were without substantial effects on cellular process outgrowth (Fig. 2bi–iii). PDGF elicited process extension in multiple cell types within the human culture system: the magnitude of effect of 100 ng/mL PDGF-AB was similar in both the Tuj1- (neuronal) and GFAP-labelled subpopulations

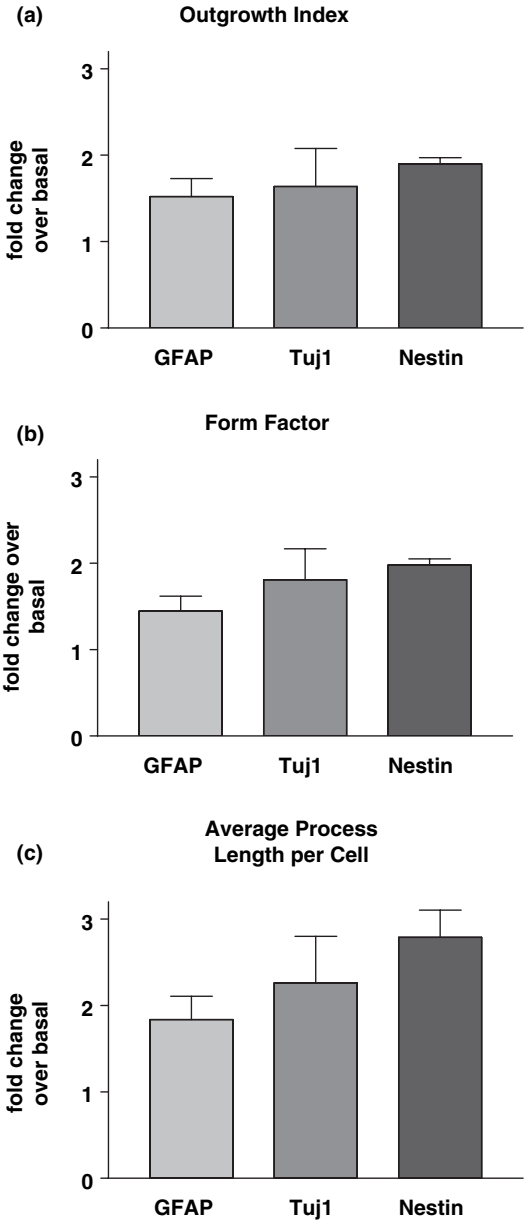

Fig. 3 100 ng/mL PDGF-AB induces similar magnitude increases in (a) outgrowth index, (b) complexity and (c) length of processes in GFAP-, Tuj1- and nestin-labelled cells ($n = 3$ separate experiments). Data are expressed as fold-change over basal.

(Figs 3a–c, $p > 0.05$ for all parameters). Data are expressed as fold-change over basal to allow for the observation that the GFAP-labelled cells tended to show higher absolute outgrowth values under both basal and stimulated conditions.

PDGF isoforms increase neural intracellular calcium

We have previously reported that PDGF is an effective activator of chemotaxis but not chemokinesis in this neural population, and that intracellular calcium, but not mitogen-activated protein kinase (MAPK), is an important regulator for chemotactic responsiveness (Richards *et al*. 2004). In order to begin to elucidate the signalling mechanisms underlying PDGF-induced outgrowth in human neural precursor cells, experiments were performed to determine the effects of PDGF on intracellular calcium levels. Using a VIPRII plate reader, 100 ng/mL of each of the three isoforms of PDGF tested evoked an increase in intracellular calcium in the human neural precursor population. The magnitude of response did not significantly vary from 1 day to 7 days in culture (Fig. 4a).

Single-cell imaging was then performed with a microscope-based imaging set-up to investigate the PDGF-induced calcium responses of the Tuj1-positive subpopulation (approximately one-third of the cells within the population). Within the Tuj1-positive subpopulations, approximately one-third of cells responded to PDGF with a calcium elevation, whereas within the Tuj1-negative population, more than half of the cells responded (Fig. 4b). Single-cell responses were characterized further using a BD Pathway HT imaging system. Using this technique, the peak calcium response to PDGF-AB had an EC_{50} of 2.33 ± 1.3 ng/mL, and the area under the curve had an EC_{50} of 2.58 ± 1.6 ng/mL (mean data from $n = 3$ experiments; greater than 20 cells per experiment; see Figs 5a and b). Calcium responses in subpopulations of the cell culture were studied by performing post hoc immunolabelling. For the three different subpopulations studied, all showed typical peak-plateau profiles (Fig. 6a), and $[Ca^{2+}]_i$ remained elevated for > 400 s. The peak amplitude and area under the curve of calcium responses to PDGF appears higher for nestin- and GFAP-positive cells than for Tuj1-positive cells (Fig. 6bi and ii; $n = 37$ GFAP+, 47 nestin+ and 9 Tuj1+ cells). However, the heterogeneity of the cell cultures is such that low numbers of Tuj1+ cells were included in this experiment and hence, it is not possible to conclude whether this represents a robust, statistically significant difference.

The PDGF-stimulated process outgrowth signalling pathway

A wide range of signalling cascades has been shown to be initiated following PDGF-receptor activation, with different pathways involved in multiple effects depending on cell type, cell cycle and duration of PDGF exposure (for reviews see Heldin and Westermark 1999; Tallquist and Kazlauskas 2004). Among the more well characterized effectors are phospholipase C (PLC)/calcium, phosphatidylinositol 3′-kinase (PI3K), cyclic-AMP responsive binding element protein (CREB), Src, MAPK and JAK/Stat. The mechanism of action of PDGF-stimulated process outgrowth in the Tuj1-positive subpopulation was investigated by incubating cells with 100 ng/mL PDGF-AB in the presence and absence of various cell signalling inhibitors.

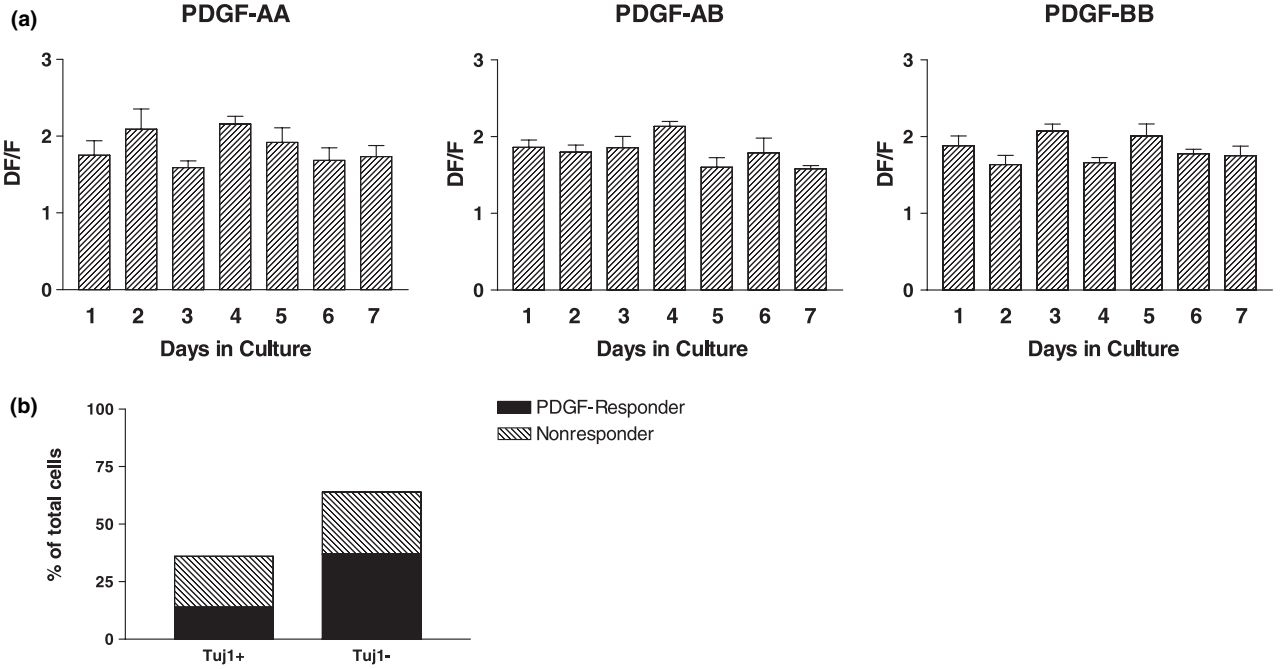

Fig. 4 (a) Calcium responses to 100 ng/mL PDGF were recorded using a VIPRII plate reader. Human neural precursors were studied 1–7 days following plating. The magnitude of PDGF-evoked responses did not vary with time between isoforms. Data are expressed as the change in fluorescence over basal (DF/F) ($n = 3$). (b) Single-cell calcium responses to PDGF-AB were measured using a microscope-based imaging system, with post hoc immunolabelling to identify cell types. Approximately one-third of Tuj1-positive cells responded with a calcium elevation, whereas more than half of the Tuj1-negative cells responded. Data are expressed as a percentage of the total number of cells studied ($n = 154$ cells).

Chelation of extracellular calcium by incubation with 2 mM EGTA abolished outgrowth responses to PDGF-AB in Tuj-1-positive cells. (Figs 7a–c). Similarly, co-application of PDGF with the JAK-3 inhibitor, WHI-P154 (30 μM), abolished PDGF-AB-induced increases in outgrowth index, process branching and process length. ($p < 0.05$, Figs 7a–c). WHI-P154 also produced a small inhibition of basal outgrowth and process length ($p < 0.05$, Figs 7d–f). In contrast, co-application of PDGF and *Clostridium* Toxin B (200 pg/mL), a selective inhibitor of Rho, Rac and Cdc-42 small GTPases, produced a small but significant increase in branching compared with PDGF alone ($p < 0.05$, Fig. 7b), whereas it had no effect on basal outgrowth (Figs 7d–f). PD098059 (50 μM), an inhibitor of MAPk kinase (Dudley *et al*. 1995), and 50 nM wortmannin, an inhibitor of PI3K (Yano *et al*. 1993), were without significant effects on either basal or PDGF-stimulated outgrowth ($p > 0.05$, Figs 7a–f).

Discussion

Neurite outgrowth is thought to be a key step in the pathway from precursor to differentiated neurone (Tojima and Ito 2004), and for establishing new synaptic contacts (Niell *et al*. 2004). In this study, a number of growth factors were investigated for their ability to produce changes in process outgrowth in human neural precursor cells. PDGF-AA, -AB and -BB were seen to produce robust increases in outgrowth in Tuj1-positive cells, not only increasing the proportion of cells with processes, but also eliciting increases in process length and complexity. Moderate outgrowth effects were elicited by bFGF, whilst aFGF and CNTF were without substantial effects in these cells. PDGF-AB was also seen to elicit process outgrowth in GFAP- and nestin-positive cells.

The importance of PDGF in oligodendrocyte proliferation and differentiation is widely accepted. PDGF has also been shown to induce neurite outgrowth in a variety of *in vitro* neuronal systems, including fetal rat and human dopaminergic neurones (Othberg *et al*. 1995), SHSY5Y cells (Hynds *et al*. 1997) and hippocampal HiB5 cells (Sung *et al*. 2001). We have previously found that PDGF increases motility in human neural precursor cells (Richards *et al*. 2004). PDGF-AA can only activate α PDGF receptor homodimers, PDGF-AB activates α homodimers or αβ heterodimers, and PDGF-BB has the broadest activity, producing αα, αβ or ββ dimers. In this study, PDGF-AA, -AB and -BB all produced similar effects on outgrowth, suggesting that receptor subtype-specific effects are not present, or not detectable, in our experimental system. However, it is possible that receptor expression and hence, isoform-specific effects, change during differentiation as has previously been shown (Erlandsson *et al*. 2001).

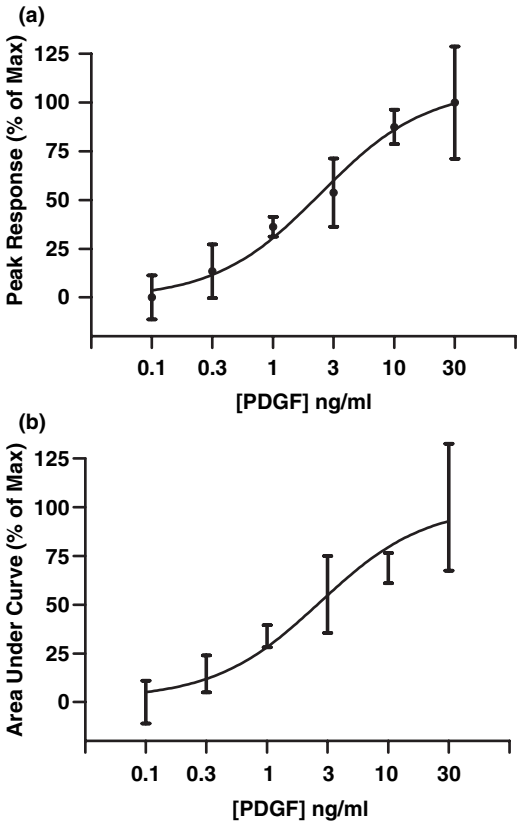

Fig. 5 Calcium responses to PDGF-AB were recorded at cell level using a BD Pathway HT imaging system. PDGF evoked a peak and plateau response, and the EC_{50} was determined for (a) the peak response (2.33 ± 1.3 ng/mL) and (b) the area under the curve (2.58 ± 1.6 ng/mL). $n = 3$ experiments, minimum of 20 cells per experiment.

Multiple signalling cascades are initiated following PDGF-receptor activation (Heldin and Westermark 1999; Tallquist and Kazlauskas 2004). Considerable cross-talk may occur between pathways, and this work has begun to elucidate which are important in PDGF-stimulated neurite outgrowth in Tuj1-positive human neural precursors. Previous studies with hippocampal HiB5 cells showed that phospholipase C (PLC) activation was critical for PDGF-induced neurite outgrowth in these cells (Sung *et al*. 2001). Activation of PLC produces an increase in intracellular calcium and activation of protein kinase C. All three isoforms of PDGF elicited an increase in intracellular calcium in subpopulations of human neuronal and non-neuronal cells, and single-cell studies showed that approximately 30% of cells positive for the neuronal pathway marker, Tuj1, responded. Additionally, 100 ng/mL PDGF-AB produces approximately a 30% increase in outgrowth index compared with basal levels, that is, approximately 30% of Tuj1-positive cells show increased outgrowth above basal levels. Whilst the experiments performed here cannot prove a direct correlation, the EC_{50} values observed for outgrowth and calcium entry were also in a similar range, suggesting that this may be a critical step in initiating outgrowth. Increased intracellular calcium has been implicated in promoting neurite outgrowth and growth cone guidance in a number of experimental systems (Lohmann *et al*. 2002; Borodinsky *et al*. 2003; Wang and Poo 2005), although it may also have a role in growth cone stalling in other circumstances (Lautermilch and Spitzer 2000). Addition of EGTA to the extracellular medium abolished PDGF-induced outgrowth in Tuj1+ cells, suggesting that an increase in intracellular calcium, more specifically, calcium influx, is critical for this pathway. With time-lapse experiments, it may be possible to directly correlate the initial calcium response with any subsequent morphological changes and cell phenotype. In this study, we have focused on the signalling pathways in Tuj1+ neural cells. However, a significant proportion of the Tuj1-negative cells responded to PDGF with increased intracellular calcium. Nestin+ and GFAP+ cells responded to PDGF with outgrowth and calcium influx, although the signalling pathways were not studied further in these cell types. In follow-up work, we are continuing to examine the mechanism of PDGF-evoked calcium responses, in particular the mechanism of calcium entry and subsequent downstream signalling cascades in both Tuj1+ and GFAP+ cells.

PI3K and the Rho-family of small GTPases have been reported to play important roles in neurite outgrowth and branching (Jackson *et al*. 1996; Leemhuis *et al*. 2004). However, in the human neural precursors, inhibition of PI3K with wortmannin did not produce significant effects on any aspect of basal or PDGF-stimulated outgrowth. Interestingly, *Clostridium* Toxin B increased the amount of PDGF-induced branching, but had no effect on basal levels. Previous reports have suggested that Rho GTPases have differing roles in axons and dendrites, with Cdc42 and Rac promoting, and Rho inhibiting, dendrite branching (Leemhuis *et al*. 2004), whilst *Clostridium* Toxin B disrupts neuronal polarization and produces cells with multiple axons in rat hippocampal neurones (Bradke and Dotti 1999). The present finding indicates a potential role for small GTPases in mediating human neural cell outgrowth, perhaps dependent on the stimulation applied.

The MAPK pathway is reported to be involved in neurite outgrowth in a variety of experimental systems (Szeberenyi *et al*. 1990; Cowley *et al*. 1994; Pang *et al*. 1995; Encinas *et al*. 1999) and may be activated downstream of PI3K (Pan *et al*. 2005) or Src (see Bromann *et al*. 2004 for review). PD098059 was without effect on PDGF-stimulated outgrowth in Tuj1-positive cells, suggesting that MAPK is not the sole effector in human neural precursors. Further experiments with combinations of inhibitors of pathways thought to work in sequence or in parallel would be necessary to confirm that PI3K and MAPK signalling are not critical in the response to PDGF.

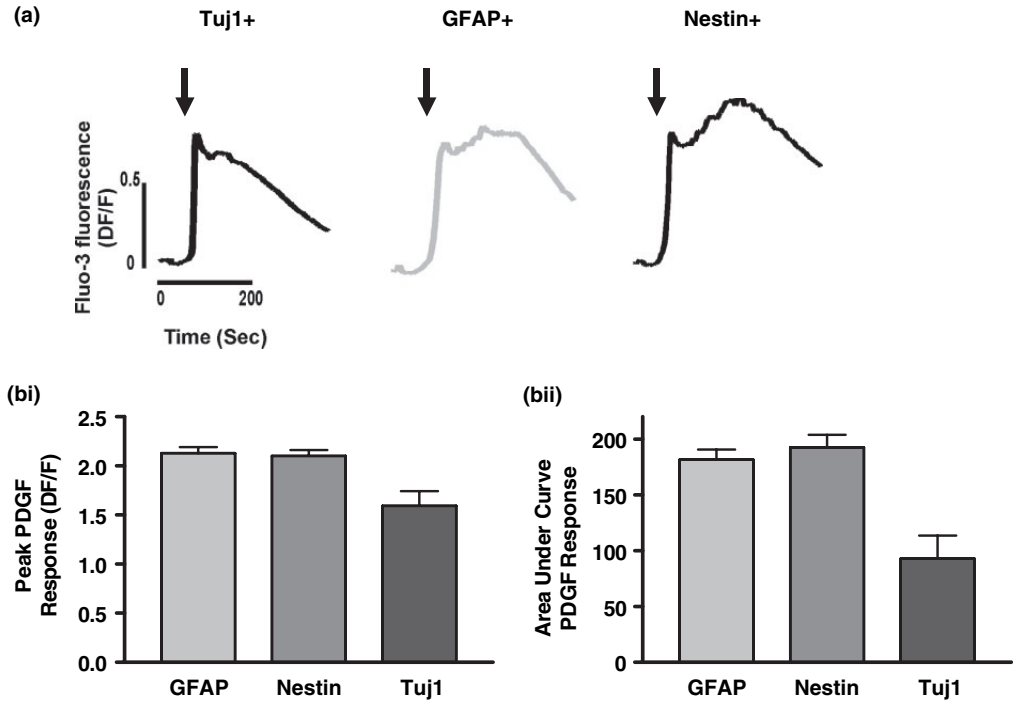

Fig. 6 Calcium responses to PDGF-AB were recorded in subpopulations of human neuronal precursors using a BD Pathway HT imaging system and post hoc immunolabelling. (a) Representative calcium traces from each cell type studied. Data are expressed as the change in fluorescence over basal (DF/F). (b) Responses to PDGF of GFAP+, nestin+ and Tuj1+ precursors, determined by both peak (i) and area under the curve (ii) measurements. Data are expressed as the change in fluorescence over basal (DF/F); only cells which produced a change of >1.1 were classified as responders and included in the analysis (n = 37 GFAP+, 47 nestin+ and 9 Tuj1+ cells).

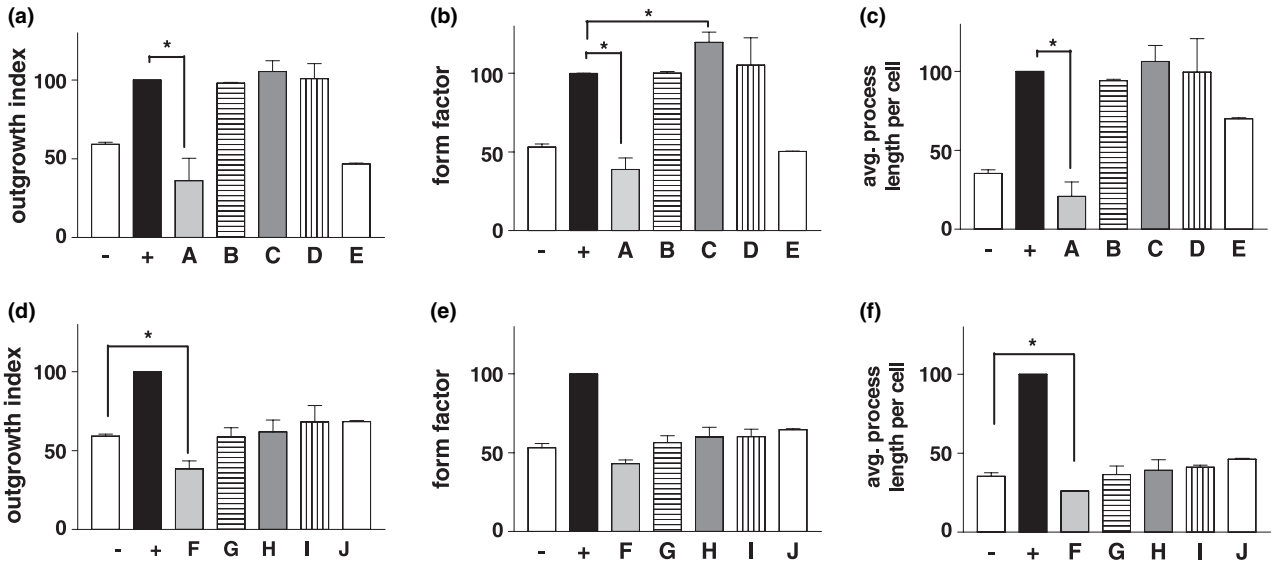

Fig. 7 The effects of various signalling pathway inhibitors on PDGF-induced (a–c) and basal (d–f) outgrowth in human neural precursors. The 100 ng/mL PDGF-AB-induced outgrowth is significantly inhibited by the JAK3 inhibitor, WHI-P154, and 2 mM EGTA. ($p < 0.05$, $n = 2$ or 3 experiments, minimum of 8 wells per experiment; $^*p < 0.05$). Abbreviations: –, basal levels; +, PDGF-AB 100 ng/mL; A, PDGF + WHI-P154; B, PDGF + wortmannin; C, PDGF + *Clostridium* Toxin B; D, PDGF + PD098059; E, PDGF + EGTA; F, WHI-P154; G, wortmannin; H, *Clostridium* Toxin B; I, PD098059; and J, EGTA. All data expressed as a percentage of the PDGF positive control response.

PDGF-induced outgrowth was inhibited by WHI-P154, a compound which is reported to inhibit JAK3 (Ghosh *et al*. 1999). This raises the possibility that JAK activation occurs upstream of calcium entry, as has been reported in mast cells (Malaviya *et al*. 1999), T cells (Säemann *et al*. 2003), and in response to chemokines (Soriano *et al*. 2003). WHI-P154 also inhibits the Src-family kinase, Hck, and the non-receptor tyrosine kinase, Syk, with similar affinity to JAK3 (Ghosh *et al*. 1999). Src and Syk are thought to be activated sequentially, and downstream targets of Syk include PLC (Sillman and Monroe 1995), PI3K (Beitz *et al*. 1999) and Vav (Deckert *et al*. 1996). Downstream targets of Vav trigger cytoskeletal reorganization (Hornstein *et al*. 2004) and have been implicated in axon guidance (Cowan *et al*. 2005). In addition, Syk may have a role in neurite outgrowth in P19 cells in response to retinoic acid or cell-matrix attachment (Tsujimura *et al*. 2001). The more selective JAK3 inhibitor, WHI-P131, could be used to define the role of JAK in this pathway. However, it is at least possible that the inhibitory effect of WHI-P154 on PDGF-induced outgrowth is in fact due to inhibition of Syk.

PDGF activates multiple signalling pathways with the considerable potential for cross-talk. For example, the signal transducer and activator of transcription (STAT) pathway has been shown to interact with the MAPK pathway (Decker and Kovarik 2000), and there is evidence for interplay between PDGF/Src/STAT signalling (Bromann *et al*. 2004). This study has highlighted the complexity of the pathways leading to neurite outgrowth, and provided initial evidence to suggest that calcium entry is critical in producing the morphological changes observed.

References

Arden S. R., Janardhan P., DiBiasio R., Arnold B. and Ghosh R. N. (2002) An automated quantitative high content screening assay for neurite outgrowth. *Chem. Today* **20**, 64–66.

Beitz L. O., Fruman D. A., Kurosaki T., Cantley L. C. and Scharenberg A. M. (1999) SYK is upstream of phosphoinositide 3-kinase in B cell receptor signaling. *J. Biol. Chem.* **274**, 32 662–32 666.

Borodinsky L. N., O'Leary D., Neale J. H., Vicini S., Coso O. A. and Fiszman M. L. (2003) GABA-induced neurite outgrowth of cerebellar granule cells is mediated by GABA(A) receptor activation, calcium influx and CaMKII and Erk1/2 pathways. *J. Neurochem.* **84**, 1411–1420.

Bradke F. and Dotti C. G. (1999) The role of local actin instability in axon formation. *Science* **283**, 1931–1934.

Bromann P. A., Korkaya H. and Courtneidge S. A. (2004) The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23**, 7957–7968.

Cowan C., Shao Y., Sahin M. *et al.* (2005) Vav family GEFs link activated Ephs to endocytosis and axon guidance. *Neuron* **46**, 205–217.

Cowley S., Paterson H., Kemp P. and Marshall C. J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**, 841–852.

Decker T. and Kovarik P. (2000) Serine phosphorylation of STATs. *Oncogene* **19**, 2628–2637.

Deckert M., Tartare-Deckert S., Couture C., Mustelin T. and Altman A. (1996) Functional and physical interactions of Syk family kinases with the Vav proto-oncogene product. *Immunity* **5**, 591–604.

Dudley D. T., Pang L., Decker S. J., Bridges A. J. and Saltiel A. R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA* **92**, 7686–7689.

Encinas M., Iglesias M., Llecha N. and Comella J. X. (1999) Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *J. Neurochem.* **73**, 1409–1421.

Erlandsson A., Enarsson M. and Forsberg-Nilsson K. (2001) Immature neurons from CNS stem cells proliferate in response to Platelet-Derived Growth Factor. *J. Neurosci.* **21**, 3483–3491.

Felling R. J. and Levison S. W. (2003) Enhanced neurogenesis following stroke. *J. Neurosci. Res.* **73**, 277–283.

Ghosh S., Zheng Y., Jun X., Mahajan S., Mao C., Sudbeck E. A. and Uckun F. M. (1999) Specificity of alpha-cyano-beta-hydroxy-beta-methyl-*N*-[4-(trifluoromethoxy) phenyl]-propenamide as an inhibitor of the epidermal growth factor receptor tyrosine kinase. *Clin. Cancer Res.* **5**, 4264–4272.

Heldin C.-H. and Westermark B. (1999) Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol. Rev.* **79**, 1283–1316.

Hornstein I., Alcover A. and Katzav S. (2004) Vav proteins, masters of the world of cytoskeleton organization. *Cell Signal.* **16**, 1–11.

Hynds D. L., Burry R. W. and Yates A. J. (1997) Gangliosides inhibit growth factor-stimulated neurite outgrowth in SH-SY5Y human neuroblastoma cells. *J. Neurosci. Res.* **47**, 617–625.

Jackson T. R., Blader I. J., Hammonds-Odie L. P., Burga C. R., Cooke F., Hawkins P. T., Wolf A. G., Heldman K. A. and Theibert A. B. (1996) Initiation and maintenance of NGF-stimulated neurite outgrowth requires activation of a phosphoinositide 3-kinase. *J. Cell Sci.* **109**, 289–300.

Lautermilch N. J. and Spitzer N. C. (2000) Regulation of calcineurin by growth cone calcium waves controls neurite extension. *J. Neurosci.* **20**, 315–325.

Leemhuis J., Boutillier S., Barth H., Feuerstein T. J., Brock C., Nurnberg B., Aktories K. and Meyer D. K. (2004) Rho GTPases and phosphoinositide 3-kinase organize formation of branched dendrites. *J. Biol. Chem.* **279**, 585–596.

Lohmann C., Myhr K. L. and Wong R. O. (2002) Transmitter-evoked local calcium release stabilizes developing dendrites. *Nature* **418**, 177–181.

Magavi S. S. and Macklis J. D. (2002) Induction of neuronal type-specific neurogenesis in the cerebral cortex of adult mice: manipulation of neural precursors *in situ*. *Dev. Brain Res.* **134**, 57–76.

Malaviya R., Zhu D., Dibirdik I. and Uckun F. M. (1999) Targeting Janus kinase 3 in mast cells prevents immediate hypersensitivity reactions and anaphylaxis. *J. Biol. Chem.* **274**, 27 028–27 038.

Mitchell B. D., Emsley J. G., Magavi S. S., Arlotta P. and Macklis J. D. (2004) Constitutive and induced neurogenesis in the adult mammalian brain: manipulation of endogenous precursors toward CNS repair. *Dev. Neurosci.* **26**, 101–117.

Niell C. M., Meyer M. P. and Smith S. J. (2004) *In vivo* imaging of synapse formation on a growing dendritic arbor. *Nat. Neurosci.* **7**, 254–260.

Othberg A., Odin P., Ballagi A., Funa K. and Lindvall O. (1995) Specific effects of platelet derived growth factor (PDGF) on fetal rat and human dopaminergic neurons *in vitro*. *Exp. Brain Res.* **105**, 111–122.

Pan J., Kao Y.-L., Joshi S., Jeetendran S., DiPette D. and Singh U. S. (2005) Activation of Rac1 by phosphatidylinositol 3-kinase

in vivo: role in activation of mitogen-activated protein kinase (MAPK) pathways and retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *J. Neurochem.* **93**, 571–583.

Pang L., Sawada T., Decker S. J. and Saltiel A. R. (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.* **270**, 13 585–13 588.

Ramos Villullas I., Smith A. J., Heavens R. P. and Simpson P. B. (2003) Characterisation of a sphingosine 1-phosphate activated Ca^{2+} signaling pathway in human neuroblastoma cells. *J. Neurosci. Res.* **73**, 215–226.

Richards G. R., Millard R. M., Leveridge M., Kerby J. and Simpson P. B. (2004) Quantitative assays of chemotaxis and chemokinesis for human neural cells. *Assay Drug Dev. Technol.* **2**, 465–472.

Säemann M. D., Zeyda M., Diakos C. *et al.* (2003) Suppression of early T-cell–receptor-triggered cellular activation by the Janus kinase 3 inhibitor WHI-P-154. *Transplantation* **75**, 1864–1872.

Sillman A. L. and Monroe J. G. (1995) Association of p72 Syk with the SRC homology-2 (SH2) domains of PLC-1 in B lymphocytes. *J. Biol. Chem.* **270**, 11 806–11 811.

Simpson P. B. (2005) Getting a handle on neuronal behaviour in culture. *Eur. Pharm. Rev.* **10**, 56–63.

Simpson P. B., Bacha J. I., Palfreyman E. L., Woollacott A. J., McKernan R. M. and Kerby J. (2001) Retinoic acid evoked-differentiation of neuroblastoma cells predominates over growth factor stimulation: an automated image capture and quantitation approach to neuritogenesis. *Anal. Biochem.* **15**, 163–169.

Soriano S. F., Serrano A., Hernanz-Falcon P., Martin de Ana A., Monterrubio M., Martinez A. C., Rodriguez-Frade J. M. and Mellado M. (2003) Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *Eur. J. Immunol.* **33**, 1328–1333.

Svendsen C. N., ter Borg M. G., Armstrong R. J., Rosser A. E., Chandran S., Ostenfeld T. and Caldwell M. A. (1998) A new method for the rapid and long-term growth of human neural precursor cells. *J. Neurosci. Meth.* **85**, 141–52.

Sung J. Y., Lee S. Y., Min D. S., Eom T. Y., Ahn Y. S., Choi M.-U., Kwon Y. K. and Chung K. C. (2001) Differential activation of phospholipases by mitogenic EGF and neurogenic PDGF in immortalized hippocampal stem cell lines. *J. Neurochem.* **78**, 1044–1053.

Szeberenyi J., Cai H. and Cooper G. M. (1990) Effect of a dominant inhibitory Ha-ras mutation on neuronal differentiation of PC12 cells. *Mol. Cell Biol.* **10**, 5324–5332.

Tallquist M. and Kazlauskas A. (2004) PDGF signaling in cells and mice. *Cytokine Growth Factor Rev.* **15**, 205–213.

Tojima T. and Ito E. (2004) Signal transduction cascades underlying de novo protein synthesis required for neuronal morphogenesis in differentiating neurons. *Prog. Neurobiol.* **72**, 183–193.

Tsujimura T., Yanagi S., Inatome R., Takano T., Ishihara I., Mitsui N., Takahashi S. and Yamamura H. (2001) Syk protein-tyrosine kinase is involved in neuron-like differentiation of embryonal carcinoma P19 cells. *FEBS Lett.* **489**, 129–133.

Wang G. X. and Poo M. M. (2005) Requirement of TRPC channels in netrin-1 induced chemotropic turning of nerve growth cones. *Nature* **434**, 898–904.

Yano H., Nakanishi S., Kimura K., Hanai N., Saitoh Y., Fukui Y., Nonomura Y. and Matsuda Y. (1993) Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* **268**, 25 846–25 856.

Zhu J., Wu X. and Zhang H. L. (2005) Adult neural stem cell therapy: expansion *in vitro*, tracking *in vivo* and clinical transplantation. *Curr. Drug Targets* **6**, 97–110.